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Development and evaluation of a TaqMan duplex real-time PCR quantification method for reliable enumeration of *Candidatus Microthrix*

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ABSTRACT

Candidatus Microtrhix parvicella is one of the most common filamentous bacteria reported to be involved in bulking and foaming problems in activated sludge plants worldwide. In order to detect and quantify both *M. parvicella* and *Microthrix calida* by quantitative PCR (qPCR), primers targeting 16S rDNA genes were designed. The qPCR reaction was optimized by using the TaqMan technology and an internal positive control was included to ensure the absence of PCR inhibitors. A total of 29 samples originating from different wastewater treatment plants were analyzed and the results were compared by using conventional microscopy, fluorescent in situ hybridization and an existing SYBR Green-based assay. Our assay showed a 100% specificity for both *M. parvicella* and *M. calida*, a sensitivity of 2.93×10^9 to 29 copy numbers/reaction, an amplification efficiency of 93% and no PCR inhibition. By performing a spiking experiment including different *Microthrix* concentrations, recovery rates ranging from 65 to 98% were obtained. A positive correlation with the SYBR Green assay ($R^2 = 0.85$) was found and most of the samples were in accordance with the microscopical observation. In comparison with SYBR Green assay, the probe-based TaqMan assay had a much lower detection limit. Compared with microscopy, some samples had a lower or higher enumeration when using qPCR. In conclusion, a qPCR method is forwarded here that could be useful as an early warning tool for fast and reliable detection of *Microthrix* in for instance sludge bulking events.

1. Introduction

Good settling properties of activated sludge are crucial for the separation of sludge from treated wastewater. However, an excessive growth of filamentous bacteria in activated sludge wastewater treatment plants (WWTPs) can cause serious operational problems (Martins et al., 2004). An overgrowth of some filaments, an event called sludge bulking, can prevent adequate flocculation and settling of the biomass. Another related problem caused by bacterial filaments is foaming, which is referring to the formation of a stable, viscous, chocolate-colored scum layer on the surface of aeration basins and secondary clarifiers (Jenkins et al., 2004). Flocs containing too many filaments are less compact and have poor settling properties. Stable foam is formed as a result of high amounts of filaments with a hydrophobic cell surface. The flocs formed by these microorganisms tend to attach to air bubbles and float on the surface of the sludge basin. Bulking and foaming sludge can therefore give rise to operational difficulties (Daims et al., 2006).

More than 30 different filament morphotypes have been described in activated sludge systems treating primarily municipal wastewater (Eikelboom, 1975, 2000) and many more are encountered in industrial treatment plant communities (Eikelboom, 2006; Eikelboom and Geurkink, 2002). Several global surveys have shown that *Candidatus Microthrix parvicella* (hereinafter referred to as *M. parvicella*) is most frequently responsible for the problems of solid–liquid separation in bulking and foaming (Jenkins et al., 2004; Martins et al., 2004; Wanner, 1994).

M. parvicella is a long, thin (diameter of 0.6–0.8 μm), non-branched and unsheathed filamentous bacterium. Its coiled appearance and characteristic Gram-positive reaction makes it possible to be recognized by microscopy (Rossetti et al., 1997). Recently, another *Microthrix* species, *M. calida*, was isolated from industrial activated sludge samples (Levantesi et al., 2006). This filament appears as a thinner version of *M. parvicella*, with 95.7–96.7% shared sequence similarity. Although there are no in situ ecophysiological studies available on *M. calida*, it is suggested that a similar behavior could be expected in pure culture and in activated sludge (Levantesi et al., 2006).

Although *M. parvicella* is infamous for causing sludge bulking and foaming, up to now it has only been detected in activated sludge systems. Some isolates have been cultured (Blackall et al., 1995, 1996; Eikelboom, 1975; Rossetti et al., 1997; Seviour et al., 1994; Slijkhuis, 1983a, 1983b; Tandoi et al., 1998; Van Veen, 1973) and only a few of them are maintained in pure culture (Blackall et al., 1995; Rossetti

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et al., 1997; Seviour et al., 1994). Its slow growth rate and difficulty in storing isolates and in generating phenotypic information have hindered attempts to elucidate the reasons for the proliferation of *M. parvicella* in WWTPs. Up to now the abundance of these bacteria is determined subjectively by observing Gram- and Neisser-stained filaments (Eikelboom and van Buijsen, 1983) or by in situ hybridization with probes targeting the 16S rDNA (Erhart et al., 1997). The first method is considered to be feasible, but requires experienced staff because of morphological variability resulting from different growth conditions (de los Reyes et al., 2002; Roels et al., 2002; Westlund et al., 1998). Yet, under those conditions a reliable identification of *M. parvicella* is possible. Low rDNA levels, because of low metabolic activity of bacteria, and a possible incomplete permeability of the cell wall for the probe can result in poor quantification of *M. parvicella* by in situ hybridization (Bradford et al., 1998; Oerther et al., 2001). A more exact quantification using quantitative PCR or qPCR (often referred to as real-time PCR) using the SYBR chemistry has recently been published (Kaetzke et al., 2005).

SYBR Green binds to all double-stranded DNA, which makes it very sensitive in the detection of nonspecific products and thus an overestimation of the target DNA. On the other hand, the TaqMan chemistry has an additional specificity as it utilizes a fluorescently labeled probe (Livak et al., 1995). In addition the use of different fluorophores enables the development of multiplex qPCR protocols whereby different targets can be co-amplified and quantified within one single reaction (Baldwin et al., 2003, 2008; Neretin et al., 2003). An example of the latter is the use of an exogenous internal positive control (IPC) (Hartman et al., 2005). In contrast to an internal inhibitor control, an IPC amplifies under the same PCR conditions as the target DNA, but using its own primer and probe sets (Hartman et al., 2005; Welti et al., 2003). PCR inhibitors have been a persistent problem when working with DNA from environmental samples. The quality of the DNA extracted from environmental samples is a limiting factor when using molecular approaches. Contaminants as such are usually inhibiting molecules that co-extract with nucleic acids (Vanysacker et al., 2010). This is particularly true when working with highly sensitive enzymatic reactions such as qPCR for which, in addition to the usual inhibition of the enzymatic reaction, environmental contaminants can also interfere with the detection method based on fluorescence (Radstrom et al., 2004; Stults et al., 2001; Wilson, 1997).

The aim of this paper was to develop a TaqMan assay for the simultaneous detection of *M. parvicella–M. calida* and PCR inhibitors. The sensitivity and specificity were evaluated and a comparison with microscopical observations and a previously published SYBR Green qPCR assay (Kaetzke et al., 2005) is discussed.

2. Materials and methods

2.1. Sampling and DNA extraction

Approximately 100 ml of activated sludge samples was taken from 29 WWTPs. Fresh samples were directly observed by microscopy and 500 μl was extracted using Mobio Ultraclean™ soil DNA kit (Cambio). In order to avoid intraspecific sample variation, each sample was extracted 4 times. Afterwards the eluted DNA was mixed together, divided in aliquot parts and stored at −20 °C until further use.

2.2. Microscopy

2.2.1. Morphological characterization

Sludge floc size and filamentous bacteria assessment were monitored by phase contrast (wet preparation) and bright field (stained preparation) microscopy using different magnifications (100×, 200×, 400× and 1000×) (Olympus BX51).

The filamentous bacteria present in WWTP were morphologically identified and the filament characteristics were determined using the Eikelboom's classification system (Table 1) (Eikelboom and Geurkink, 2000; Eikelboom and van Buijsen, 1983). Referring to morphological and color characteristics of the filaments (Jenkins et al., 2004), the Gram staining and Neisser staining were respectively performed as described by Adamse (1970) and Gurr (1965). The morphological characterization and quantification were performed at the laboratory of Aquafin N.V. The laboratory is accredited by the Belgian Accreditation Body as a testing laboratory, according to the requirements of NBN EN ISO/IEC 17025:2005 for the determination of filamentous bacteria in wastewater samples. The filament abundances were categorized in 4 groups: low, moderately, highly and very highly concentrated.

2.2.2. Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed according to a protocol slightly adapted from Nielsen (2009). The EUB338 probe was used for the hybridization of all bacteria (Amann et al., 1990) and the MPA-MIX probe for *M. parvicella* and *M. calida* (Erhart et al., 1997) (Table 2). Slides were mounted in Citifluor (Citifluor Ltd.) and visualized with a BX51 epifluorescence microscope (Olympus) equipped with a UPlanApo 100x/1.35 oil objective, U-M51009 filterset and Cell P software (Olympus).

2.3. Design of oligonucleotides, DNA cloning and sequencing

For the TaqMan assay, a new primer–probe set for the quantification of *M. parvicella* 16S rRNA gene was designed, relying on an alignment of different *M. parvicella* 16S rRNA sequences (accession numbers X89774, X89561, X89560 and X82546) (Table 2). Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA) following the user manufacturer guidelines for the design of MGB probes. To confirm the specificity of the primers and probes, the PCR product was cloned using the TOPO® TA Cloning® Kit (Invitrogen) and blasted against the non-redundant GenBank database (http://blast.ncbi.nlm.nih.gov). Clones were generated by inserting the PCR products, derived from the new designed primer pair and activated sludge samples as template, together with the pCR™ 2.1-TOPO® vector into One Shot® *Escherichia coli* cells according to the manufacturer's instructions. Colonies that contained a plasmid with an insert were screened on Lysogeny Broth agar plates supplemented with ampicillin (50 μg/ml, Sigma-Aldrich). Positive colonies were selected and controlled by PCR. The PCR mixture contained 1 μl of DNA, 1 μl $MgCl_2$ (50 mM), 2.5 μl of 10× reaction buffer (Invitrogen), dNTPs (2 mM), 0.1 μl *Taq* polymerase (2.5 U) and 1 μl of M13 forward (5′-GTAAAACG ACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers (20 mM). PCR fragments of successfully cloned vectors were collected, purified using Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare), and sequenced using the BigDye Terminator Sequencing kit v3.1 (Applied Biosystems). The sequencing reaction was performed with M13 forward and reverse primers separately. Thermal cycle conditions were: initial denaturation at 95 °C for 1 min, followed by 25 cycles of 95 °C for 10 s, 50 °C for 10 s, and 60 °C for 4 min. The resulting products were sequenced by capillary electrophoresis on an ABI Prism 3130-Genetic Analyzer (Applied Biosystems) using POP7 polymer. Consensus sequences as determined with accompanying software (SeqScape v2.5) were aligned using BioEcovit v7.1.9.

2.4. Preparation of the calibration standards

Since no pure culture of *M. parvicella* was available, a calibration standard was constructed using amplified PCR-fragments. The most convenient way to create a DNA standard is to clone a PCR product into a standard vector. Advantages of this method are: large amounts of standard can easily be produced, the ability of species identification by sequencing, and the possibility of DNA quantification by spectrophotometry. The previously described clones (Section 2.3.), where after the M13 PCR and the purification step, were quantified using Quant-iT™

Table 1
Results of filament identification, filament- and floc characteristic determined by microscopy. "+" means that the particular species was present. Types 0041, 0092, and 1851 refer to Eikelboom's types 0041/0675, 0092 and 1851 respectively. The parameters were evaluated using Eikelboom's classification system (17, 32).

Sample number	Filament identification						Filament characteristic		Floc characteristic	
	M. parvicella	Type 0041	Type 0092	*Nocardia* spp.	*Nostocoida limocola*	Type 1851	Abundance	Effect on floc structure	Shape	Structure
1	+						Very high	Cluster–cluster aggregation	Irregular	Diffuse
2	+	+					High	Bridging	Irregular	Diffuse
3	+	+		+			High	Open floc structure	Irregular	Diffuse
4	+				+	+	High	Bridging	Irregular	Diffuse
5	+	+					High	Cluster–cluster aggregation	Irregular	Compact
6		+					High	Open floc structure	Irregular	Diffuse
7	+	+					High	Bridging	Agglomerates	Diffuse
8	+	+					High	Open floc structure	Irregular	Diffuse
9							Moderate	Little or none	Round	Compact
10	+	+					High	Open floc structure	Agglomerates	Diffuse
11	+	+					High	Cluster–cluster aggregation	Irregular	Diffuse
12	+	+					Moderate	Open floc structure	Irregular	Diffuse
13	+						High	Cluster–cluster aggregation	Irregular	Diffuse
14	+	+					High	Open floc structure	Irregular	Diffuse
15	+	+					High	Open floc structure	Irregular	Diffuse
16	+	+					Moderate	Little or none	Irregular	Diffuse
17							Moderate	Open floc structure	Irregular	Diffuse
18	+	+					High	Cluster–cluster aggregation	Round	Compact
19	+	+					Moderate	Open floc structure	Irregular	Diffuse
20	+						Very high	Cluster–cluster aggregation	Irregular	Diffuse
21	+	+					High	Bridging	Agglomerates	Diffuse
22	+						Very high	Bridging	Agglomerates	Diffuse
23	+	+	+				Very high	Cluster–cluster aggregation	Agglomerates	Diffuse
24							Moderate	Open floc structure	Irregular	Diffuse
25	+	+		+			High	Cluster–cluster aggregation	Round	Compact
26	+			+			Moderate	Open floc structure	Irregular	Diffuse
27	*M. parvicella* + *calida*		+				High	Open floc structure	Irregular	Diffuse
28	+	+	+		+		High	Little or none	Round	Compact
29	+	+	+				High	Cluster–cluster aggregation	Irregular	Diffuse

PicoGreen® dsDNA Assay Kit (Invitrogen). The exact amount of DNA was calculated as follows: (X (g/μl DNA)/[plasmid lenght in bp × 660]) × (6.02×10^{23}) = Y amplicon/μl. Based on the assumptions that the average 16S rDNA gene copy number is 3.05 for the Actinobacteria related species, the relative abundance of *Microthrix* cells was recalculated (rrndb release 3.1.221 Klappenbach et al., 2001).

2.5. Quantitative PCR

2.5.1. Optimization reactions

First, the qPCR protocol was optimized using different probe types, primer concentrations, and MM (MM) reagents.

Considering the probe design, a Minor Groove Binding (MGB) and a TAMRA probe with slightly different sequences were compared. The TAMRA probe contained a 6-carboxy-tetramethyl-rhodamine quencher molecule and a longer sequence length (23 bp). The MGB probe was shorter (16 bp) and contained a non-fluorescent quencher.

Secondly, the optimal primer concentrations were determined in separate tubes by running a matrix of five different forward and reverse primer concentrations (500, 250, 100, 75 and 10 nM) according to the Application note of Applied Biosystems (136AP04-01; http://www.appliedbiosystems.com).

Finally, three types of MM were tested: TaqMan® Gene expression MM (P/N 4369016, Applied Biosystem, CA, USA), TaqMan® Fast Advanced MM (P/N 4444557 Applied Biosystem, California, USA) and qPCR MM Plus (P/N 05-QP2X-03-075, Eurogentec, Seraing, Belgium). The MM was evaluated in terms of amplification efficiencies and background noise in the non-template control.

2.5.2. qPCR reaction

DNA amplification and PCR product detection were performed using the ABI Prism® 7000 Sequence Detection System (Applied Biosystems). The amplification efficiencies were evaluated using a TaqMan® Exogenous Internal Positive Control (IPC) (Applied Biosystems) (Hartman et al., 2005). Each sludge sample was assayed together with their 10-fold dilution in triplo. A 25 μl qPCR reaction containing 2.5 μl of template DNA, 12.5 μl of 2× qPCR MM Plus (Eurogentec), 75 nM of forward and reverse primers and 0.2 μM of the MGB labeled probe

Table 2
Primers and probes used in this study.

Target group	Use	Type	Dye	Name	5′ to 3′ sequence	Reference
Bacteria	qPCR	Forward primer		BACT1369F	CGGTGAATACGTTCYCGG	Suzuki et al. (2000)
		Reverse primer		PROK1541R	AAGGAGGTGATCCRGCCGCA	
		MGB probe	FAM™	TM1389F	CTTGTACACACCG GCTGCCTC	
					CCGTAGGAGTCCCGTC	
	FISH	FISH Probe	Cy3	EUB338	GCTGCCTCCCGTAGGAGT	Amann et al. (1990)
M. parvicella	qPCR	Forward primer		S-S-M.par-0828-S-21	GGTGTGGGGAGAACTCAACTC	Kaetzke et al. (2005)
		Reverse primer		S-S-M.par-1018-A-17	GACCCCGAAGGACACCG	
M. parvicella and *M. calida*	qPCR	Forward primer		S-G-Mtx-0939-a-S-20	GCAACGCGAAGAACCTTACC	This study
		Reverse primer		S-G-Mtx-1012-a-A-19	AGCCATGCACCACCTATCG	
		MGB™ probe	FAM™	S-G-Mtx-0985-a-S-16	CAGAGATGCGGTGTCC	
	FISH	FISH probe	FITC	MPA-MIX (MPA60 + MPA223 + MPA645)	GGATGGCCGCGTTCGACT	Erhart et al. (1997)
					GCCGCGAGACCCTCCTAG	
					CCGGACTCTAGTCAGAGC	

(Applied Biosystems) (Table 2). The IPC was co-amplified in each PCR reaction, according to the manufacturer's instructions. Double distilled water was used as a negative control. The cycle program was: 2 min at 50 °C, 10 min at 95 °C, and followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The standard curve was automatically generated by the ABI Prism system by plotting the cycle threshold (C_t), at which the threshold fluorescence was reached, versus the logarithmic concentration of positive control DNA. The C_t is defined as the number of cycles required for the fluorescent signal of the target DNA to cross the threshold (i.e. exceeds background signal). C_t levels are inversely proportional to the amount of target nucleic acid in the sample. Efficiencies were calculated from the equation $E = [(10^{-1\ /\ slope}) / 2] \times 100\%$ (using the slope of the serial dilution's standard curve). qPCR data analysis was performed with the SDS 1.2.3. software (Applied Biosystems). PCR inhibition was determined by comparing the amplification plot of the IPC of the samples with the amplification plot of the IPC of the positive control DNA. As previously mentioned, IPC amplifies under the same PCR conditions as the target DNA but by using its own primer and probe sets (Hartman et al., 2005), meaning that the target DNA amplification efficiency will never be affected by competition of both IPC and target DNA for the same primer and probe sets. As PCR inhibition mostly concerns inhibition of the DNA polymerase (Stark et al., 2000), the IPC and the target DNA are exposed to the same degree of amplification inhibition. For the enumeration of the entire bacterial population, universal primer and probe targeting highly conserved regions of the 16S rDNA gene were used (Table 2). The results of the new developed TaqMan assay were compared with a published SYBR Green assay for the specific detection of *M. parvicella* (Kaetzke et al., 2005). Amplification reactions were performed, as described by Suzuki et al. (2000) and Kaetzke et al. (2005) respectively.

2.6. qPCR recovery rates

To determine whether a known quantity of added DNA can be successfully assayed against a background of other DNA from a range of other organisms a "spiking experiment" was performed. For this purpose three samples were evaluated by microscopy and scored as low, moderately or highly concentrated with *Microthrix*. After the DNA was extracted and spiked with four different concentrations of clonal *Microthrix* DNA. The spiked samples were then quantified by qPCR and recovery rates were calculated.

2.7. Determination of PCR inhibition by polyaluminium chloride

Polyaluminium chloride (PAX) treatments have been proved to be an effective measure to suppress *Microthrix* growth in WWTP (Nielsen et al., 2005; Paris et al., 2005). To exclude possible PCR inhibition by PAX, an additional inhibition experiment was performed using different PAX-14 concentrations. Briefly, 0, 150, 250, 500, 800 and 1000 μl/l (i.e. respectively 0, 7, 12, 23.5, 37.6 and 47 g Al^{3+}/kg MLSS) PAX-14 was added to 6 randomly chosen activated sludge samples. Two experimental conditions were tested: the samples were mixed and incubated 15 min or 2 h. Subsequently, the DNA of 500 μl of homogenized sludge was extracted and further analyzed by TaqMan qPCR.

2.8. Data analysis

The data from the optimization experiments were compared using one-way analysis of variance (ANOVA) test (Statistica 11.0).

3. Results and discussion

3.1. Determination of the TaqMan assay specificity and optimization

In order to determine the specificity of the new developed primers, the generated PCR product was sequenced and verified on NCBI (http://blast.ncbi.nlm.nih.gov). The most relevant results are shown in Table 3. Our assay shows a 100% specificity for both *M. parvicella* and *M. calida*. Sequences of accession numbers DQ147284.1 and DQ147282.1 showed respectively one gap and one insert in their original sequence. For another sequence (accession number X89560.1) 98% similarity was found due to two unspecified basepairs within the original sequence. Compared to the assay developed by Kaetzke et al. (2005), *M. calida* has been included in the TaqMan assay, as it is difficult to make a distinction between the two related species. Only slightly thinner filaments and a weaker reaction to Gram-staining characterized the latter (Fig. 4). The comparison between qPCR and microscopy is described in detail in Section 3.3. As the TaqMan assay was developed for both *M. parvicella* and *M. calida*, from now on the term "*Microthrix*" will be used to specify the detection of both morphotypes.

The qPCR protocol was optimized using different probe types, primer concentrations, and MM reagents. In comparison with the MGB assay, the TAMRA probe had a lower PCR efficiency (89.21 vs 93.86%) (Fig. 1). Moreover, the background signal emitted from the quencher molecule of the TAMRA assay makes low concentrated samples difficult to quantify. Such a signal contamination was noticed at 30 and 34 cycle times for the TAMRA and the MGB assay respectively. The background component of the no template control (NTC) was mathematically removed by the software algorithm of the real-time cycler. The discussed results below are based on the MGB assay.

Five different primer concentrations were tested on *Microthrix* clonal DNA and DNA extracted from activated sludge samples. The optimal primer concentration was 75 nM as higher concentration (500, 250, 100 nM) had similar C_t values and lower concentration (10 nM) results in a weak signal and lower C_t values.

Table 3
Most relevant NCBI accession numbers, names and relevant parameters after BLAST of sequenced PCR product using the new developed TaqMan primers and probes.

Accession	Name	% identity	Mismatches	Gap opens	E-value	Bit score
DQ147281.1	*Candidatus Microthrix calida* strain TNO1-1 16S ribosomal RNA gene	100	0	0	3.00E−40	171
DQ147277.1	*Candidatus Microthrix calida* strain TNO2-1 16S ribosomal RNA gene	100	0	0	3.00E−40	171
DQ147283.1	*Candidatus Microthrix calida* strain TNO2-3 16S ribosomal RNA gene	100	0	0	3.00E−40	171
DQ147284.1	*Candidatus Microthrix calida* strain TNO2-4 16S ribosomal RNA gene	99	0	1	5.00E−38	163
FJ638889.1	*Candidatus Microthrix parvicella* clone M2 16S ribosomal RNA gene	100	0	0	3.00E−40	171
JQ624332.1	*Candidatus Microthrix parvicella* clone OTU-5-40m.ABB 16S ribosomal	100	0	0	3.00E−40	171
DQ147278.1	*Candidatus Microthrix parvicella* strain BIO17-1 16S ribosomal RNA	100	0	0	3.00E−40	171
DQ147279.1	*Candidatus Microthrix parvicella* strain BIO17-2 16S ribosomal RNA	100	0	0	3.00E−40	171
DQ147282.1	*Candidatus Microthrix parvicella* strain BIO17-3 16S ribosomal RNA	99	0	1	1.00E−38	165
DQ147280.1	*Candidatus Microthrix parvicella* strain EU18 16S ribosomal RNA	100	0	0	3.00E−40	171
X93044.1	*M. parvicella* 16S ribosomal RNA.	100	0	0	3.00E−40	171
X82546.1	*M. parvicella* 16S rDNA gene.	100	0	0	3.00E−40	171
X89561.1	*M. parvicella* DNA for 16S ribosomal RNA gene (clone 17).	100	0	0	3.00E−40	171
X89560.1	*M. parvicella* DNA for 16S ribosomal RNA gene (clone 6).	98	2	0	5.00E−38	163
X89774.1	*M. parvicella* DNA for 16S ribosomal RNA gene.	100	0	0	3.00E−40	171

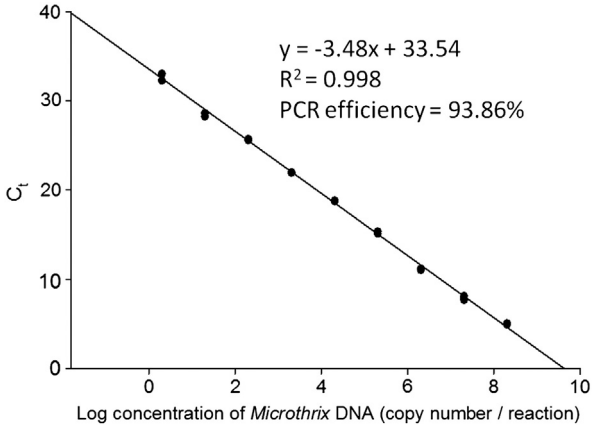

Fig. 1. Serial 10-fold dilution from 2.93×10^9 to 29 copy numbers/qPCR reaction of *Microthrix* DNA. The standard curve was generated by a linear regression of the threshold cycle (C_t) versus the logarithm of the *Microthrix* DNA concentration per qPCR reaction.

Considering the MM type, no significant differences in PCR efficiencies were found ($p = 0.64$). On the other hand significantly lower NTC was found using the MM Plus (C_t 36.35 ± 1.72) compared to the gene expression (C_t 34.24 ± 1.20) and fast advanced MM (C_t 31.62 ± 0.08) ($p = 0.008$). Although higher NTC values were found using the 16S rDNA assay (ranging from 23.74 to 28.95), no significant differences were found between the tested MM ($p = 0.14$). The MM effect was more pronounced in the total bacteria 16S rDNA assay. Including the consideration of the price of each TaqMan qPCR run, being respectively 2.14, 2.58 and 1.48 Euro per run for the MM Plus, the TaqMan® Gene Expression Master Mix and the TaqMan® Fast Advanced Master Mix, the MM Plus has been selected based on the observation of lower NTC values and equal PCR efficiencies compared to the other MM types.

3.2. Determination of the TaqMan assay sensitivity

Plasmids containing cloned *Microthrix* 16S rDNA were used to generate a standard curve relating C_t to the number of gene copies. Serial 10-fold dilutions ranging from 2.93×10^8 to 29 copy numbers/qPCR reaction, which is equivalent to 4.15×10^4 fg to 4.15×10^{-3} fg DNA, were amplified (Fig. 1). To determine the precision of the TaqMan assay, C_t values for five replicates of tenfold dilutions of cloned *Microthrix* 16S rDNA were measured (Table 4). The results represent independent dilution series and different PCR runs. The mean C_t values, standard deviation, and percent CV (coefficient of variation) were calculated for each dilution. The results showed low variability, with CVs ranging from 0.23 to 2.85%.

To determine the assay sensitivity, a 'spiking experiment' was conducted and qPCR recovery rates were measured (Table 5). The qPCR was not able to recover 100% of the *Microthrix* DNA. The mean recovery rate was 81.45 ± 10.31% (ranging from 66 to 98%). Every sample type yielded a lower concentration than expected. This effect was more pronounced for low concentrated samples. This is probably due to differences in amplification efficiency (clonal vs genomic DNA), primer/probe competition, etc. In addition, the spiking experiments revealed that there was no PCR inhibition (IPC C_t values varied between 28.6 and 29.7), meaning that the IPC assay is functioning properly to assess inhibition. Spiking with high DNA concentrations (>10 ng DNA) reveals IPC C_t values higher than 30 or IPC inhibition. This inhibition is due to competition between the internal control with the target, when this target is present in excess. Comparable results have been described by the IPC manufacturer and in the literature (Behets et al., 2007; Hallett and Bartholomew, 2009; McBeath et al., 2006). Similar values have been found by performing the same spiking experiment using a singleplex approach (i.e. *Microthrix* without IPC) (data not shown).

Table 4
Summary of five different PCR runs performed on eight separate DNA dilution series of *Microthrix* 16S rDNA.

Number of copies/PCR reaction	Mean C_t	SD[a]	CV[b] (%)
2.93×10^8	4.99	0.068	1.36
2.93×10^7	7.88	0.224	2.85
2.93×10^6	11.15	0.089	0.80
2.93×10^5	15.26	0.128	0.84
2.93×10^4	18.77	0.051	0.28
2.93×10^3	21.96	0.049	0.23
2.93×10^2	25.64	0.113	0.44
2.93×10^1	28.42	0.275	0.97

[a] SD: standard deviation of five replicates.
[b] CV: coefficient of variation.

Table 5
Results of spiking experiments. BD: below detection limit, '/' means that no spiked DNA was added.

Sample type	Spiked amount	Obtained value	Expected value	Recovery
Low concentrated Microthrix sample				
Initial 16S concentration: $1.11 \pm 0.1 \times 10^4$	/	BD		
Initial *Microthrix* concentration: BD	2661.81	2332.55 ± 365.67	2661.81	88%
	266.18	176.58 ± 18.23	266.18	66%
	26.62	21.52 ± 4.72	26.62	81%
	2.66	BD	2.66	
Moderately concentrated Microthrix sample				
Initial 16S concentration: $2.56 \pm 0.8 \times 10^5$	/	263.33 ± 42.63		
Initial *Microthrix* concentration: 263.33 ± 42.63	2661.81	2304.03 + 187.37	2924.81	79%
	266.18	427.67 ± 71.27	529.51	81%
	26.62	212.89 ± 39.80	289.95	73%
	2.66	172.51 ± 21.18	265.99	65%
Highly concentrated Microthrix sample				
Initial 16S concentration: $1.04 \pm 0.8 \times 10^7$	/	1195.31 ± 156.98		
Initial *Microthrix* concentration: 1195.31 ± 156.98	2661.81	3287.72 ± 687.45	3857.12	85%
	266.18	1316.55 ± 343.30	1461.49	90%
	26.62	1106.74 ± 120.09	1221.93	90%
	2.66	1178.76 ± 365.49	1197.97	98%

3.3. Comparison between TaqMan, SYBR Green and microscopy

Twenty-nine samples were processed and examined for the presence of *M. parvicella* and *M. calida* using the new described TaqMan assay, the previously described SYBR Green assay (Kaetzke et al., 2005) and conventional microscopy (Fig. 2). The results obtained with the TaqMan assay revealed no PCR inhibition in any sample as determined by the IPC controls (C_t values: 28.22 ± 0.48).

Microthrix was detected in all the samples (Fig. 2), which is not surprising as it is not only most frequently responsible for the problems of solid–liquid separation in bulking and foaming (Rossetti et al., 2005) but like many other filamentous species, it also forms an essential part of the floc structure (Sezgin et al., 1978).

In every case, the TaqMan assay detected more cells compared to the SYBR Green. Although the differences were small in most cases, some low concentrated samples showed a pronounced assay effect (samples 3, 17 and 24). The correlation of the *Microthrix* counts was examined by comparing the linear relationship between both detection methods on the 29 samples (Fig. 3). A clear positive correlation between the 2 assays was found ($R^2 = 0.85$). Similar as in Fig. 2, the highest variability is attributed to the samples with a low concentration of *Microthrix*. In contrast to what occurred in the SYBR Green-based quantification, the signal generation in the TaqMan assay was strictly specific due to cleavage of the TaqMan probe. Moreover the inter-assay variation of the SYBR assay was much higher than the TaqMan assay. NTC values

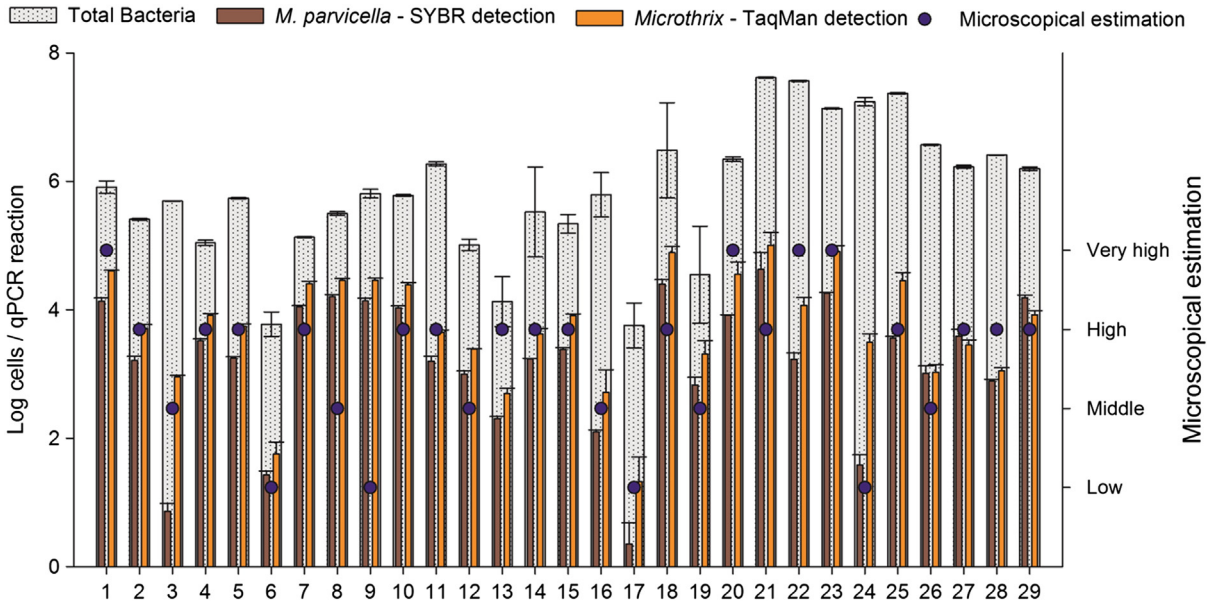

Fig. 2. Amount of total bacteria (gray, dotted) enumerated by 16S qPCR, *M. parvicella* enumerated with SYBR Green qPCR (dark colored) and *Microthrix* enumerated with TaqMan qPCR (light colored) of the 29 analyzed samples. The cell densities are expressed in log values/qPCR reaction (left axis). Mean values ± standard deviation values for triplicates are shown. Microscopic quantification based on Eikelboom's classification system is plotted with blue dots (right axis). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

varied between C_t values of 24.86–28.01 and 32.25–38.58 for the SYBR and TaqMan assay respectively. Also the melting temperatures in the SYBR assay varied between 83.8 and 85.0 °C. Therefore, it is suggested that the TaqMan assay offers a more accurate quantification of low copy numbers compared to the SYBR Green based quantification.

The 29 samples were also compared with the microscopic data. In general, ten samples had a higher estimation using qPCR and 3 samples (13, 22, 28) showed a higher *Microthrix* concentration using microscopy. Referring to the qPCR assays, ten samples showed a high similarity against the TaqMan assay, whereas 4 samples matched slightly better with the SYBR Green enumeration. Interestingly, in some cases the amount of total bacteria and *Microthrix* seems to be correlated (Figure 2, samples 6 and 17), yet a low correlation was found for both SYBR Green ($R^2 = 0.158$) and TaqMan ($R^2 = 0.175$) enumerations when related to the total bacteria counts. Therefore to avoid artificial values we plotted both enumerations on one graph instead of the *Microthrix*/total bacteria ratios like done by Kaetzke et al. (2005) and Kumari et al. (2009) (Fig. 2).

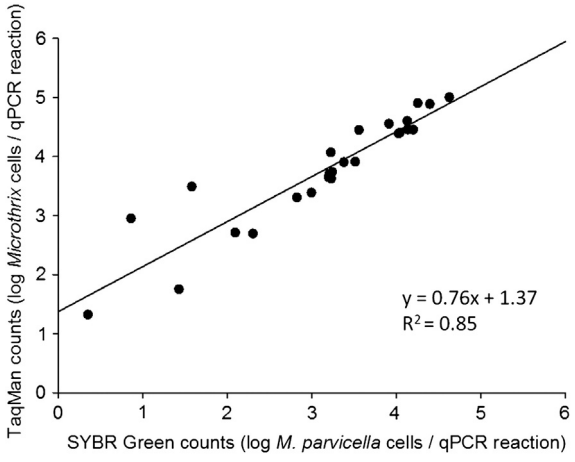

Fig. 3. Correlation between SYBR Green and TaqMan quantification for 29 sludge samples.

As mentioned above, the TaqMan assay was designed for both *M. parvicella* and *M. calida* morphotypes. Interestingly, the different morphotypes were found in sludge collected from the same sample (sample 27, Figs. 4E and F, 5B). One filament had a thickness in the range of 0.59–0.74 μm and was identified as *M. parvicella* (Fig. 4A), whereas another filament was clearly thinner (0.27–0.32 μm) and recognized as *M. calida* (Fig. 4B and C). Yet, no differences were found between the TaqMan and the SYBR Green assay for this particular sample, meaning that the differences are depending more on the sensitivity of the qPCR chemistry than on the primer specificity. Using FISH, Levantesi et al. (2006) found that the smaller filament diameter is not always a reliable distinctive character for in situ identification. Indeed, it was found that both thin and normal *Microthrix* filaments gave a positive signal with the Mpa-all-1410 probe suggesting that these unknown *M. parvicella* filaments were responsible for settling problems in several plants (Levantesi et al., 2006).

As the MPA-MIX probe was used in this study, no distinction could be made by FISH analysis (Fig. 5B). Therefore it is important to monitor different *Microthrix* morphotypes like suggested in this study. The fact that different morphotypes coexist in the same environment suggests that they are using different niches. Indeed, Levantesi et al. (2006) found that *M. calida* is able to grow at higher temperature and pH and no growth of *M. calida* is observed on *M. parvicella* specific medium (Slijkhuis, 1983a, 1983b). On the other hand pure culture studies indicate a lipase activity and a preferentially incorporation of long-chain fatty acids as an organic carbon and energy source, which is similar to *M. parvicella*.

Samples with a higher and lower estimation were further analyzed in detail. Samples 13, 22 and 28 had a lower estimation using qPCR. The IPC was never inhibited meaning that no PCR inhibitors were present. As different floc structures (compact (Fig. 5C) and diffuse (Fig. 5A and B)) were found, it can be excluded that the DNA extraction method was not able to degrade compact sludge flocs completely. Moreover, the DNA extraction method has been previously evaluated for different sludge types and should normally give an optimal DNA recovery (Vanysacker et al., 2010). Some samples were higher estimated using qPCR. However, for most of them the differences were rather small (samples 7, 10, 12, 18, 26) (Fig. 2). In contrast, sample 8 was 100 times higher estimated using qPCR. This sample had been subjected

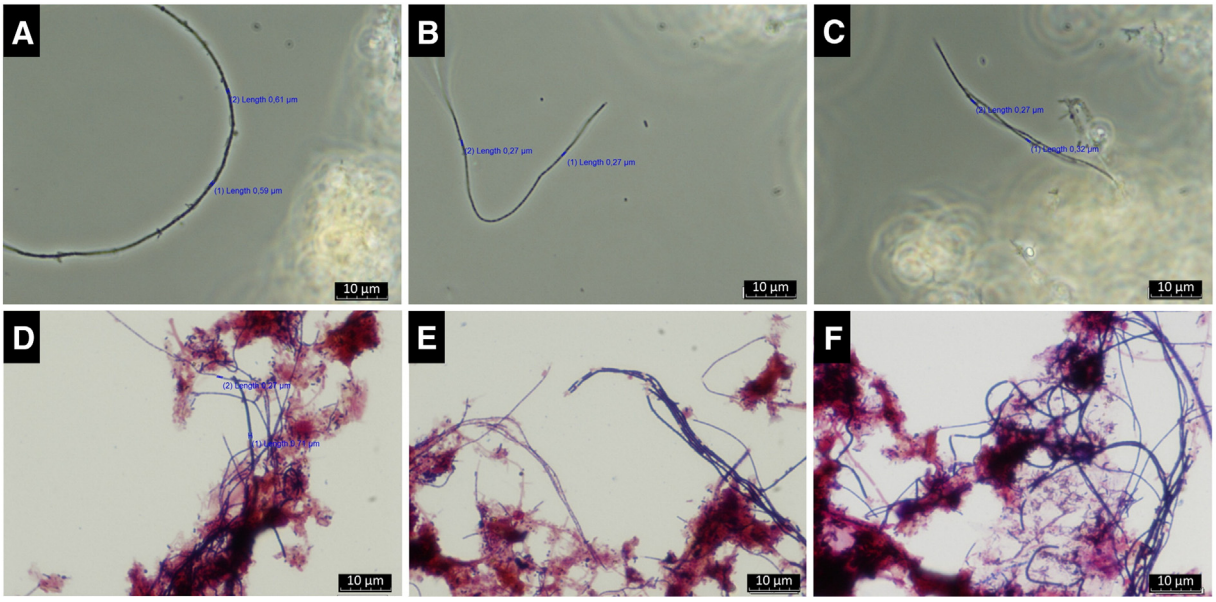

Fig. 4. Phase-contrast (A, B, C) and Gram-staining (D, E, F) micrographs of a sample containing the two *Microthrix* morphotypes (sample 27). Thin filament morphotypes presumably *M. calida* are shown in B and C. The *M. parvicella* morphotype is visible in A, D, E, and F and shows the occurrence of both filaments in the same sample.

to a PAX treatment. The exact effect of PAX on *M. parvicella* is not really clear, yet a change in surface properties and a decreased in fatty acid transport have been observed, followed by a decrease competitiveness of *M. parvicella* to the other bacteria (Hamit-Eminovski et al., 2010). Roels et al. (2002) observed a filament reduction only after 3 weeks PAX dosing whereas the floc structure started to improve after 10 to 15 days. In such situations *Microthrix* fragments are much shorter and difficult to detect with microscopy, but still present in the sample leading to a high qPCR enumeration. In addition, when the PAX treatment in such situations was stopped, a fast recovery of *Microthrix* and thus sludge bulking occurred (personal observation). These observations could explain why lower estimation of *Microthrix* was found using microscopy when compared to qPCR. A similar phenomenon has been observed for sample 21 (Fig. 2).

To exclude possible PCR inhibition by PAX for these two samples, an additional inhibition experiment was performed using different PAX-14 concentrations. In all cases, no PCR inhibition was found (IPC values in the range of 27.5–30.9, detailed data not shown). This means that PAX did not affect the qPCR reaction and rather differences between microscopy and qPCR were due to small, difficult to detect, *Microthrix* fragments.

The strong differences found in sample 9 remain unexplained (Fig. 2). As the amount of filament was low, no filament-identification could be done (Table 1). Samples 5, 9, 18, 25 and 28 were characterized by compact flocs (Fig. 5C), however this type of flocs did not lead systematically to a higher estimation by qPCR (samples 9, 18, and 25 versus 5 and 28). Also we could not explain the discrepancy between the SYBR Green and TaqMan results for sample 24 (Fig. 2). Some samples were distinct by clusters of Gram-positive cells, however these clusters did not affect the enumeration and the FISH staining (sample 26, Fig. 5A).

To summarize, this paper describes a method for the simultaneous detection of the *M. parvicella* and *M. calida* 16S rDNA gene and an IPC using the TaqMan chemistry. The additional TaqMan specificity afforded not only a higher accuracy compared to SYBR Green, but also the possibility of using multiple TaqMan probe and primer sets at the same time (Baldwin et al., 2003, 2008; Neretin et al., 2003). Moreover, using mulitplex PCR, simultaneous detection of total Bacteria or other problematic species such as Eikelboom's types (021N, 0675, 0041, 0961, 1701, 0914 and 0092), *Thiothrix eikelboomii*, *Nostocoida limocola*, and *Gordonia amarae, etc.* would be possible (Dumonceaux et al., 2006; Jenkins et al., 2004; Marrengane et al., 2011; Nielsen et al., 2009; Seviour et al., 1994; Vervaeren et al., 2005). However, the development of a multiplex qPCR using the same gene is difficult due to the competition for resources. Moreover, when one target is more abundant than another, unequal amplification will occur and the differences will be enhanced during each cycle, leading to artifact and misinterpretation of the data (Edwards and Gibbs, 1994). Furthermore, the use of the 16S rDNA gene, even though it is a highly homologous target sequence for practically all bacteria which can be used for detection (Barken et al., 2007; Mothershed and Whitney, 2006), can deliver some technical problems. Firstly, bacterial groups have different 16S rDNA copy numbers, ranging from 1 to 13, making the comparison between distinct species difficult (Fogel et al., 1999). Secondly, the main problem for detection of bacterial DNA is the achievement of a high sensitivity along with a clear negative control. Up to now, this is an unresolved problem since the reagents used in the PCR process are contaminated with trace amounts of bacterial DNA, especially the *Taq* polymerase, where contamination originates from its production in bacterial cultures (Philipp et al., 2010). Therefore, the use of functional genes for multiplex PCR seems to be much easier. Recently, a genome announcement of *M. parvicella* has been published (Muller et al., 2012). Although the sequence assembly is not complete yet, some functional gene sequence will be soon available and the subsequent development of other quantification tools will be possible.

4. Conclusions

M. parvicella is one of the most common filamentous bacteria reported to be involved in bulking and foaming problems in activated sludge plants worldwide. Therefore an identification and quantification of this microorganism is a prerequisite for developing efficient control strategies to predict bulking and foaming events. The discussed duplex qPCR assay for the detection of *Microthrix* is a fast, accurate and sensitive surveillance tool with a much faster turnaround time than the classical microscopy. Making use of the TaqMan chemistry, a good correlation with an existing SYBR Green assay and conventional microscopy was demonstrated. Moreover, using TaqMan, a lower detection limit and lower NTC values were found compared to the SYBR Green assay. Although no PCR inhibition was found, the routine use of an IPC is

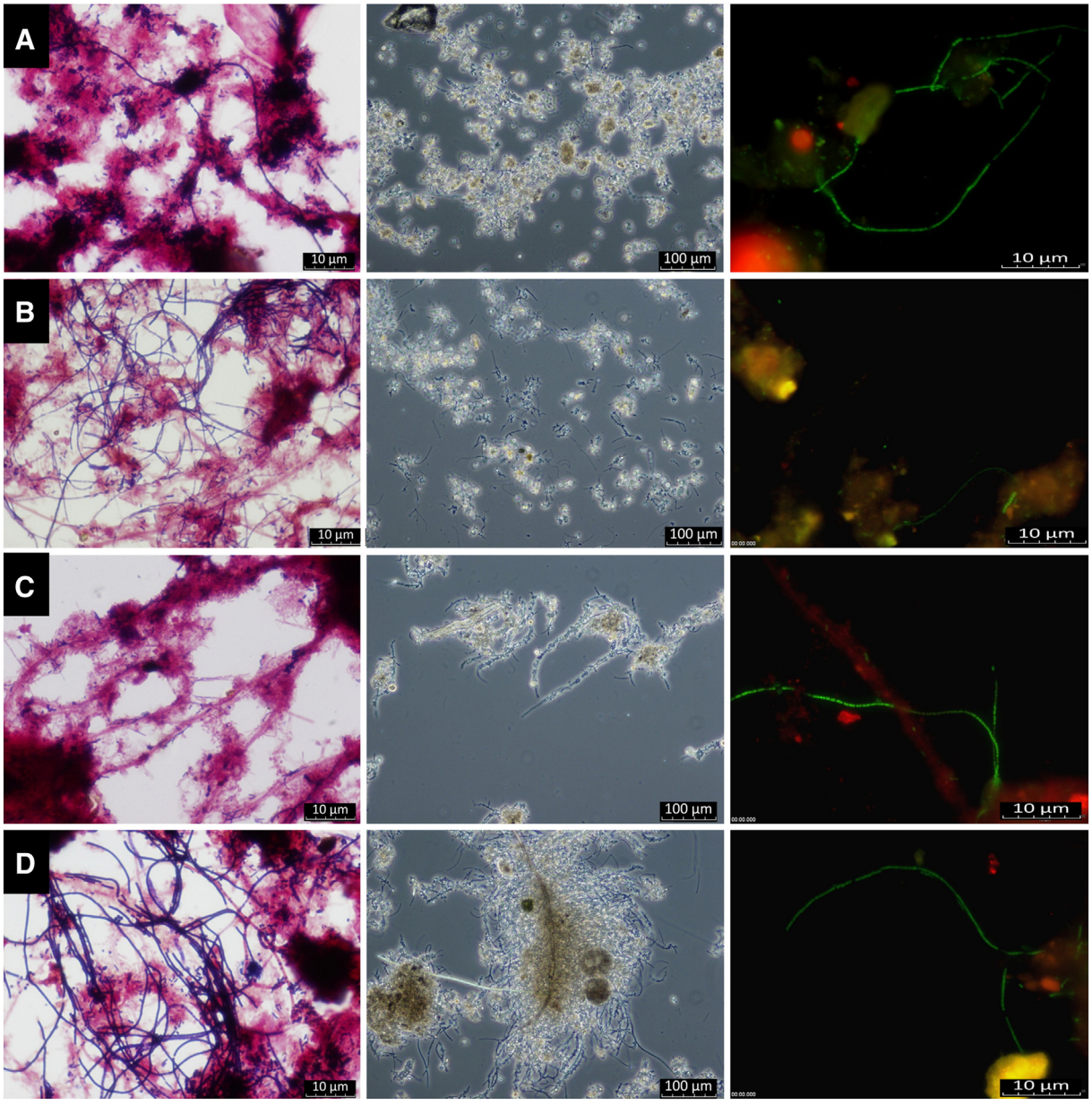

Fig. 5. Gram-staining (left), phase-contrast (middle) and FISH staining (right) of samples 26 (A), 27 (B), 28 (C) and 29 (D). FISH staining is performed with the MPA-MIX (FLUOS, green) and EUB (Cy3, red) probes detecting both *M. parvicella* as *M. calida*. Sample 26 (A) is characterized by a moderate amount of *M. parvicella* and aggregates of Gram-positive cells. Samples 27 (B), 28 (C) and 29 (D) contain a high amount of *M. parvicella*.

strongly recommended to detect inhibitors during DNA amplification as it remains unpredictable whether environmental samples contain inhibiting substances.

Acknowledgments

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